REMARKS

Status of the Claims

Claims 33 and 34 are pending. Claims 33 and 34 currently stand rejected. Claims 33 and 34 are identical to issued claims 1 and 33, respectively, of U.S. Patent No. 6,162,258 to Scarborough, *et al.* (the "Scarborough patent").

Rejection of Claim 34 under 35 U.S.C. §102(b)

Claim 34, which is identical to claim 33 of the Scarborough patent, was rejected under 35 U.S.C. §102(b) as anticipated by Livesey, *et al.* (U.S. 5,336,616).

In the Office Action, it is contended that Livesey ('616) teaches contacting a bone with glycerol and freeze-drying it. Col. 3 lines 31-37 and 53-57 of Livesey ('616) were cited. Applicant respectfully disagrees.

In the background section of Livesey ('616) at col. 3 lines 31-37, it is taught that allogenic bone may be used fresh or may be cryopreserved (e.g., frozen not freeze-dried) with DMSO and/or glycerol, to maintain cellular components. According to Livesey ('616) "It is thought that the cellular components contain histocompatibility antigens, and are capable of eliciting an immune response from the host. In many cases, the patient receiving the allogenic transplant undergoes immunosuppressive therapy." (See col. 3 lines 38-42.) Thus, Livesey ('616) understands that an immune response may be induced by tissues cryopreserved with DMSO and/or glycerol and therefore teaches away from the use of cryoprotectants.

Also in the background section of Livesey ('616) at col. 3 lines 53-57, Livesey ('616) goes on to teach that **alternative** methods (see col. 3, line 50) to the cryopreservation techniques using DMSO and/or glycerol taught at lines 31-37 have been developed. Livesey ('616) states,

"Alternative processing methods have been developed by others that are intended to address the limitations of allogenic and animal-derived transplant tissues. Freeze-drying is used routinely in the processing of allogenic bone for transplantation. It has been found that the freeze drying process results in a graft which elicits no significant rejection response as compared to fresh or cryopreserved allogenic bone." (See col. 3 lines 50-57.) Cells of allografts freeze-dried without cryoprotectants (such as glycerol or DMSO, among others) are destroyed during the freeze-drying process. It has been observed that, as a result, these bone allografts do not elicit a specific immune response. (See Schwade, N., Implants, Soft Tissue, Alloderm, emedicine.com, copyright 2004; Kreuz, F., *et al.*, The Preservation and Clinical Use of Freeze Dried Bone, J Bone Joint Surg. 1951; 33A: 863, which are attached.)

It is known in the art that cryoprotective agents like DMSO and glycerol improve the survival rate of cells that are frozen by decreasing the temperature at which ice forms. The movement of water into and out of the cell during chilling determines the dynamics of intracellular ice formation and cell survival. Livesey ('616) teaches that surviving cells can elicit an immune response, and that known freeze drying processes result in grafts which elicit no significant rejection response. As such, Livesey ('616) does not teach bone being lyophilized in the presence of glycerol or another "liquid organic preservation solution." Withdrawal of the rejection of claim 34 is respectfully requested.

Rejection of Claim 34 under 35 U.S.C. §102(e)

Claim 34, which is identical to claim 33 of the Scarborough patent, was rejected under 35 U.S.C. §102(e) as anticipated by Boyce, *et al.* (U.S. 5,899,939). However, Boyce ('939) does

not teach a method for producing a bone graft as in independent claim 34 of the present application.

Penetration and retention of a liquid organic material (e.g., plasticizer) in a bone during lyophilization does not result in a change in the orientation of the collagen fibers in the bone. (See page 10 lines 21-23.) In the specification of the present application, it is taught that, "In fresh bone, water serves a solvating function in bone tissue allowing proper orientation and molecular spacing of the collagen fibrils which maintain structural alignment of the mineral phase in association with the organic phase." (See page 2 lines 27-29.) In the specification of the present application, it is further taught that removing the water from fresh bone results in alteration of the molecular structure of the bone tissue and as a result of the reorientation of the collagen fibrils. (See page 3 lines 2-5.)

The only reference to freeze-drying in the Boyce ('939) patent is in Example 1, col. 6 lines 48-52. In the example, a cortical section of bone was continuously wetted with water, while layers were cut from the bone. The layers of the bone were then frozen to -70°C and freeze-dried for 48 hours. Water was, thus, removed, and a liquid organic material did not remain in the bone during its lyophilization, as in claim 34. Water is not a liquid organic material. Without the use of a liquid organic material as in the claimed invention, the orientation of the collagen fibers was altered. Boyce ('939) does not teach bone grafts having an unaltered orientation of collagen fibers, as in the claimed invention. Applicant respectfully requests that the rejection of claim 34 of the present application be withdrawn.

DOCKET NUMBER: 64230-00005USD2
PATENT

Rejection of Claim 33 under 35 U.S.C. §103(a)

Claim 33, which, as stated above, is identical to claim 1 of the Scarborough patent. For the reasons set forth above and in the November 15, 2004, Amendment, it is asserted that Livesey ('616), Boyce ('939), and Morse, *et al.* (U.S. 5,333,626) taken alone or in combination do not teach every element of the claimed invention. Withdrawal of the rejection of claim 33 is respectfully requested.

Conclusion

In view of the foregoing, the application is respectfully submitted to be in condition for allowance, and prompt favorable action thereon is earnestly solicited.

If there are any questions regarding this amendment or the application in general, a telephone call to the undersigned would be appreciated since this should expedite the prosecution of the application for all concerned.

If necessary to effect a timely response, this paper should be considered as a petition for an Extension of Time sufficient to effect a timely response; please charge any deficiency in fees or credit any overpayments to Deposit Account No. 10-0447 (64230-00005USD2).

Respectfully submitted,

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Synonyms and related keywords: acellular dermis, allograft skin, cross-linked porcine skin, allograft rejection, transplantation, cadaveric skin, cadaveric skin, acellular matrix, acellular human dermis

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INTRODUCTION Section 2 of 6 Back Top Next
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Throughout history, multiple approaches have been used to replace lost, damaged, or diseased tissues. Some methods use synthetics, biosynthetic constructs, cross-linked biological materials, or preserved allografts. Investigations using these approaches have supplied increasing evidence that the matrix component of tissue replacement must be complex. The physician must understand the normal structure of the integument and how this arrangement is either preserved or slightly altered by the fabrication methods of these allograft materials. These concepts become increasingly important when considering the remodeling of the allograft material after implantation into the patient. Factors to be considered by the physician while selecting the allograft material include the patient's medical history, the anatomic site of implantation, the desired result, and the chosen material type.

Integument structure

Skin consists of 2 essential layers: the epidermis, which provides a barrier against the environment, and the dermis, which provides strength, durability, and elasticity. The dermis of the skin is a complex multicomponent matrix. In contrast to skin of other species, human skin lacks a panniculus, or underlying muscle sheath. Because many allograft materials are xenografts, this anatomic fact is important to remember. Consequently, the human dermis has evolved as a multilayered complex organ able to deal with the stress patterns resulting from the relative immobility of human skin. The layers of the dermis include the papillary dermis and the reticular dermis. The reticular dermis transitions into the deep reticular-subcutaneous junction.

The important components of the dermis that contribute to its function include the basement membrane complex at the dermal-epidermal junction, collagen, elastin, proteoglycans, and a distinctive vascular plexus. The basement membrane complex at the dermal-epidermal junction contains type IV collagen, laminin, and highly specialized type VII collagen. Type VII collagen forms anchoring fibrils and filaments, which ensure strong physical bonding of the epidermis to the dermis.

The bundle orientation of collagen and elastin differs between the papillary dermis and the reticular dermis. Collagen bundle orientation is random in the papillary dermis, but it is perpendicular to the lines of tension in the deeper reticular dermis. Similarly, elastin fibers are sparse and finely reticular in the papillary dermis, whereas they are thicker and form a complex 3-dimensional array in the reticular dermis.

The dermal vasculature forms a distinct plexus in the papillary dermis. This plexus configuration plays an important role in the remodeling process because collagen deposition tends to occur along the pathways of neovascularization. If the plexus is absent, collagen remodeling occurs along the pathways of an altered vascular pattern, as evident in granulation tissue in scar formation. Proteoglycans of the dermis provide a reservoir for growth factors (eg, basic fibroblast growth factor [FGF] binds to heparin sulfate). Dermal proteoglycans also direct the assembly of collagen (eg, decorin, tenascin) or are involved directly in angiogenesis and the regulation of cellular functions (eg, hyaluronic acid, chondroitin sulfate). Each of these factors plays a significant role in the ability of the allograft material to maintain volume persistence over time.

Dermal function depends on the intricate and complex organization of the extracellular matrix components and their interactions. Matrix components include collagen fibrils oriented perpendicular to the lines of stress, an elastin network consisting of elastin and microfibrillar fibers, diverse and compartmentalized proteoglycan species, and a structurally unique basement membrane complex.

Wound healing

Following full-thickness skin injury, the epidermis heals rapidly by regeneration. However, the dermis is not capable of regeneration and, therefore, heals by repair.

This repair process begins with the formation of granulation tissue, which subsequently matures into th scar tissue. This process often leads to disfigurement and functional impairment of the integument. Scar tissue is different from the dermis becaus it is a r latively simple undifferentiated structure consisting of parallel arrays of collagen bundles that orient parallel to the lines of stress. Scar tissue also lacks elastin, normal distribution of proteoglycans, and the organs of the normal dermis.

Because of the limited supply of human donor tissue, an ideal approach to replacing lost dermis is to use a synthetic or biosynthetic material. Allograft skin and cross-linked porcine skin have been used as temporary wound dressings, but they cannot provide a permanent dermal replacement because they are either rejected or do not revascularize.

Allograft rejection

The cause of allograft rejection is a subject of continued research and controversy. The use of allograft donor skin as a permanent skin replacement in full-thickness injuries is limited by its immunogenic properties. Allograft skin grafts routinely incorporate to a full-thickness wound, but they ultimately are rejected. This immune response to allograft skin is directed primarily against the cells of the epidermis and endothelial and fibroblast cells of the dermis. The noncellular components of the dermis, consisting primarily of extracellular matrix proteins and collagen, have been demonstrated to be relatively nonimmunogenic. Before the existence of AlloDerm, the difficulty involved in removing the immunogenic cells from the nonimmunogenic dermis of allograft skin restricted its use to temporary coverage of full-thickness burns.

The concept of permanent transplantation of nonimmunogenic extracellular tissue matrix has been illustrated clearly with the more robust matrix of bone. According to Kreutz, freeze-dried bone allografts have been used successfully in permanent bone implants for many years. The cells in these allografts are destroyed during the freeze-drying process, but the structural organization of the extracellular matrix remains intact. These bone allografts do not elicit a specific immune response, and they provide an environment of osteogenic cell repopulation and osteoinduction with subsequent matrix remodeling of a new bone formation. The success of this product illustrates the advantage of supplying a matrix that is remodeled later into normal tissue (see Image 1).

Kreutz reaches lyoph. w/o cryoprotectant

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AlloDerm is fabricated by a proprietary method of processing cadaveric skin. This method produces an acellular dermis that is free of the cells responsible for the antigenic response to allograft skin. After processing, the skin is reduced to a basement membrane and properly oriented dermal collagen matrix. The end result is an acellular human dermis that theoretically will not be rejected.

Tissue banks procure fresh human cadaveric skin following American Association

of Tissue Bank (AATB) guid lines. All donor m dical history and serologic screening are performed in accordance with th AATB and Food and Drug Administration (FDA) guidelines. All tissue is tracked from donor to recipient, and relat d sampl s are archived.

Aseptic processing then is performed by a patented method (see Image 2). The processing of the dermis involves removal of the epidermis under tonic conditions that induce separation of the anchoring fibrils from the hemidesmosomes of the basal keratinocytes. The fibroblasts then are extracted from the dermal matrix under conditions that do not alter the collagen bundles or damage the basement membrane complex. The resulting acellular matrix then is cryoprotected. Cryoprotection elevates the glass transition temperature of the matrix to a level compatible with conventional freeze-drying temperatures. The cryoprotected matrix then is frozen in a lyophilizer and dried with a 2-step drying procedure.

Matrix integrity is assessed throughout the process by electron microscopy to verify retention of the basement membrane complex, normal collagen bundle, and binding patterns during removal of cellular material (see Image 3). All samples are processed with an accompanying satellite sample.

Following full processing, all satellite samples are reassessed for microbiological culture. Any bacterial growth is regarded as contamination and results in failure to obtain quality assurance release. One of the steps used in the AlloDerm process has been demonstrated by an independent contract laboratory to inactivate a concentrated suspension of HIV. Although it does not ensure viral sterility, it represents an added safeguard.

AlloDerm is supplied to physicians in packages consisting of a sealed foil bag that contains the material in an inner peel pouch. Each package of AlloDerm contains one piece of freeze-dried acellular human dermis. Rehydration instructions, the product insert, and the tissue transplant return record are attached to the bag. The sheet material is available in many different sizes (see Image 4).

POTENTIAL USES IN OTOLARYNGOLOGY	Section 4 of 6 [Back Top Next]
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The following briefly describes some of the many uses of AlloDerm in otolaryngology and head and neck surgery. Listing all the potential uses of this unique material is not possible; however, a basic overview is provided. This information is not designed to be a protocol or specific instructions for the implantation of this material. As always, the physician should exercise individual judgment regarding the possible uses of AlloDerm. The basic benefits that apply to all applications include the following:

- The tissue is nonreactive, and the graft remodels like autogenous tissue.
- No migration occurs, and the graft integrates into surrounding tissue.
- After rehydration, AlloDerm is pliable and can be cut, folded, or rolled.

- The material is strong and sutures like tissu .
- Only 10 minutes of rehydration are r quired.
- The material eliminates donor site trauma.
- Volume persistence is maintained during the remodeling period of the integument.

A large body of literature demonstrates the utility of AlloDerm for the treatment of full-thickness burns. This topic is beyond the scope of this manuscript; however, the reader is referred to excellent journal articles written on the subject (see Bibliography). Moreover, authors such as Schulmann provide significant documentation about the use of AlloDerm in the gingiva. AlloDerm can be used in patients of any age; physicians successfully have treated patients aged 2-80 years using this material.

Parotidectomy

Replacing large volumes of isolated soft tissue following oncologic surgical resection remains a challenge to the surgeon. Very few synthetic or semisynthetic materials have been effective for long-term reconstruction. Traditionally, large defects have been filled with autogenous tissue, which must be harvested in either a vascularized or nonvascularized form. This process extends operating time and often results in postoperative morbidity. Synthetic biomaterials, such as expanded polytetrafluoroethylene sheeting, avoid the problems of autogenous grafts, but they are not ideal. The risk of eventual infection or extrusion always is present, even years after implantation.

Implantation of the AlloDerm graft for large-area defect repair usually is accomplished with the patient under general anesthesia. Based on the size of the defect requiring correction, either the 2X4-cm or the 3X7-cm size of thick AlloDerm graft can be selected. The AlloDerm easily can be rehydrated in 2 washes of sterile isotonic sodium chloride solution for a minimum of 5 minutes per wash. Prophylactic intravenous antibiotic may be administered 30 minutes prior to incision at the discretion of the surgeon. Several sheets of the AlloDerm graft then may be trimmed and stacked into the defect to replace the inadequate soft tissue.

The sheets may be sutured together to aid placement and prevent shifting during the immediate postoperative period. A slightly larger sheet then may be used to cover the stacked sheets placed in the cavity, thereby providing a single smooth surface underneath the patient's skin to minimize the potential for cutaneous irregularity over the implantation site. The most superficial AlloDerm sheet (which also should be the largest in surface area) can be notched around its perimeter to feather the edge and may facilitate graft integration within the surrounding soft tissue.

Care also should be taken whenever possible to secure the edges of this AlloDerm top sheet at multiple points around its perimeter with a 4-0 clear nylon suture.

Meticulous hemostasis is important prior to closure to prev nt an excess collection of blood and seroma formation. A small suction drain should be placed into th surgical cavity before wound closure and exited through a separate stab wound. The us of suction drainage is strongly recomm nded when multiple she ts of AlloDerm are stacked and implanted.

Lip augmentation

A number of case studies concerning the use of AlloDerm in lip augmentation and reconstruction have been presented. Several examples presented in the literature use the sheet material and obtain excellent cosmetic results and little loss of volume at follow-up care. A patient undergoing revision surgery after an evulsion injury is an excellent candidate for an AlloDerm reconstruction. After rehydration, the pliability of AlloDerm allows individual shaping of the graft contour to best fit the defect. This allows for excellent customization (eg, having minimal increase in lateral quadrants with increased bulk at midline).

One example of the surgical procedure using the sheet AlloDerm graft for lip augmentation usually can be performed with a local anesthetic block on an outpatient basis. Horizontal stab incisions are made with a No 11 blade at the vermilion border in the lateral commissure of the lip. The plane superficial to the orbicularis oris muscle is identified using blunt scissors for approximately 1-1.5 cm. Blunt dissection along the vermilion border in the submucosal/supra orbicularis oris muscle plane may be performed using a canthal awl, creating a tunnel connecting the lateral stab incisions.

The AlloDerm graft then is rehydrated in sterile isotonic sodium chloride solution, and the resulting soft pliable material may be trimmed to the appropriate dimensions with scissors. The AlloDerm then may be rolled upon itself to form a cylinder, and interrupted 4-0 chromic sutures may be used to secure the roll. The graft then may be affixed to the end of a canthal awl and pulled through the previously created submucosal tunnel. After appropriate positioning, the graft then may be sutured in place with 2 interrupted 4-0 chromic sutures placed in the submucosal plane at the commissures. A recent study has demonstrated that the use of AlloDerm in the aforementioned fashion yields a superior result compared to the use of autologous fat.

Nasal septum perforation

A detailed description of the surgical procedure for nasal septal perforation is beyond the scope of this manuscript. Different operative plans are used depending on the size of the defect. Small nasal septal perforations usually are described as those less than 2 cm while large defects are greater than 2 cm. The surgical technique used for closures of small nasal septal perforations consists of an extended external rhinoplasty approach with elevation, rotation, and meticulous suture closure of the perforation. Bilateral intranasal mucosal flaps with a posteriorly based pedicle are used. AlloDerm material has been used as an interpositional graft between the septal flaps to repair the defect and eliminate the need to obtain an autograft.

After exposure of the septal defect, an AlloDerm graft is secured to the septal cartilage with 5-0 Vicryl sutures superior to the perforation and allowed to drap ov r and completely cover th perforation on one side of the septal cartilage. The mucoperichondrial flap then is laid into position over the top of the AlloDerm graft. The remaining edges of the graft and flap are secured with sutures. This procedure then is repeated on the contralateral side of the nasal septum. A similar procedure is used to correct large nasal septal perforations using AlloDerm material. The main difference is that this procedure typically is performed in 2 stages, which usually requires tissue expansion to produce flaps large enough to cover the increased area.

Nasal reconstruction

A traumatic nasal deformity can be corrected using a combination of AlloDerm and cartilage. AlloDerm has been used as an onlay graft to restore contour to a saddle deformity, and it has been used in conjunction with the autogenous auricular cartilage to form a premaxillary implant. Dorsal correction and contour have been maintained 1 year after surgery, and no evidence of resorption was present in the areas where AlloDerm was used to replace damaged tissue. Importantly, AlloDerm is used as a scaffold and bulking agent but does not provide structural support.

Rhytid revision

AlloDerm implantation also can help treat premature onset of dermal atrophy leading to inadequate integument and a perioral disfigurement. This particular revision can be accomplished in the office with the patient under local anesthesia. The AlloDerm sheet material may be rolled and implanted in the perioral area using a procedure similar to that described for lip augmentation.

Depressed scar revision

AlloDerm also has been successful in procedures to revise depressed scars in the midface area. AlloDerm was used in conjunction with a W-plasty to reconstruct areas of depressed and hypertrophied scars. The scar may be excised along its entire course. When excising the scar, a W-plasty may be performed and the triangular flaps may be interdigitated and closed over the AlloDerm graft. The rehydrated graft may be folded over to create the appropriate size before insertion into the subcutaneous area. Significantly improved cosmetic result has been noted at 8 and 10 months postoperatively, which indicates that this contour probably is maintained throughout the remodeling period.

Other uses

Other suggested uses for AlloDerm include plastic surgery of the eyelid and orbit, repair of the dura, tympanic membrane reconstruction, and effacement of nasal labial folds.

Micronized AlloDerm

Cymetra recently has been approv d by the FDA for use in soft tissue augmentation. Injectable AlloDerm obviates the need for incisions and surgical dissection; additionally, the injectable material allows for more precise placement of the implant.

Other injectable materials are available; the most commonly used is collagen. This has various applications, both cosmetic and reconstructive. Processed type I bovine collagen (Zyderm) has been used successfully since the late 1970s. This material attempts to correct dermal deficits with xenograft dermal proteins. Its major drawback is loss of volume persistence over weeks to months. In an attempt to solve this problem, the manufacturer produced Zyplast, which is a type I bovine collagen cross-linked with glutaraldehyde. Zyplast lasts longer than non–cross-linked Zyderm, although both materials eventually are resorbed completely. Allergic reactions have been reported from both products in 3% of patients.

Injectable suspensions that consist predominantly of type I collagen (Autologen, Dermalogen) also are used to fill soft tissue defects. Autologen is composed of autologous collagen extracted from skin procured from the patient during previous elective surgery. Dermalogen is similar, except the skin is obtained from tissue banks and processed routinely. The major drawbacks to Autologen are the requirement of the previous surgical procedure to acquire skin and a processing time of 3-4 weeks; with Dermalogen, the collagen may be damaged during the processing, increasing its antigenicity.

The injectable form of AlloDerm, Cymetra, recently has been made available for use. Micronized AlloDerm is created by homogenizing an AlloDerm sheet cut into strips. This homogenizing process is performed at very low temperatures, preserving both the basement membrane and the integrity of the collagen fibers. The product is injectable-sized particles of AlloDerm that maintain the ultrastructure of the dermis and can easily pass through a 26-gauge needle. A clinical study describes the biological behavior and clinical effect of subdermally implanted AlloDerm versus injected micronized AlloDerm. The results of this experiment showed great promise for the use of micronized AlloDerm in facial plastic surgery.

Other extracellular matrix material

Small intestinal submucosa (SIS) is a collagen-based extracellular matrix in current clinical use. It is a sterile acellular graft material extracted from the small intestine of pigs using proprietary processing methods and marketed under the trade names of Oasis, Surgisis, and Stratasis by COOK. A rapidly growing base of clinical experience with this material shows it has potential in many reconstructive applications.

Another material that has been introduced recently is Dermaplant, which is another allogenic dermis available in sheets. Little information is available regarding the clinical use of Dermaplant.

Dynamic improvement continues in the types of materials available for soft tissue augmentation, and many future studi s are required to learn more about the long-

term characteristics of each.

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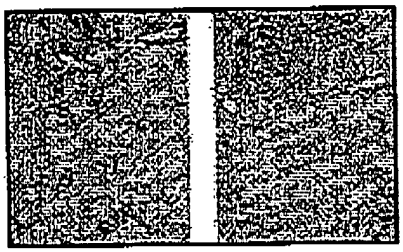
Caption: Picture 1. AlloDerm implants. A human biopsy sample 15 days postsurgery. Comparison of the control site (left) and the AlloDerm site (right) reveals minimal histologic differences. Analysis of the AlloDerm site reveals infiltration of host fibroblasts and evidence of neovascularization.



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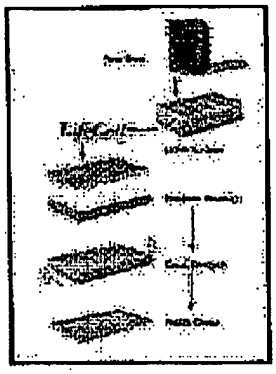
Picture Type: Photo

Caption: Picture 2. AlloDerm implants. Schematic of AlloDerm processing.



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Picture Type: Image

Caption: Picture 3. AlloDerm implants. Electron micrographs of allograft skin (left) and AlloDerm (right). The AlloDerm shows intact collagen fiber bundles (CF), elastin (E), and space previously occupied by a dermal fibroblast (arrows). The bars represent 5 micrometers.

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eMedicine Zoom View (Interactive!)

Picture Type: Photo

Caption: Picture 4. AlloDerm implants. Rehydrated AlloDerm.

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Picture Type: Photo

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THE PRESERVATION AND CLINICAL USE OF FREEZE-DRIED BONE *

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Orthopaedic casualties have once again become a serious problem. The bone-graft surgery necessary for proper rehabilitation promises to be extensive in many cases. The use of preserved grafts as a means of shortening operative time and convalescence, minimizing surgical shock, and decreasing postoperative pain is receiving careful consideration in selected cases. Bone banks utilizing freezing temperatures and chemicals for preservation are the most popular. However, the expense of freezing equipment, the difficulty in shipping, the change of chemicals, and the frequent cultures necessary for aseptic control combine to make the availability of suitable preserved bone a serious military problem.

Since the economic disadvantages of storage of bone by freezing seem to be paralleled by subsequent clinical disadvantages, it was determined to seek a better method of preservation. It was noted that freeze-drying answered many requirements. The virtue of drying from the frozen state seems to be derived from two factors:

1. Quick freezing at very low temperatures minimizes protein denaturation;

2. Drying from the frozen state lessens harmful concentration of salts as the solid state is maintained throughout dessication[1].

Products which are preserved by drying from the frozen state can be stored at room temperatures and therefore may be shipped in convenient packages without refrigeration. Organic materials such as freeze-dried plasma, commonly known as "lyophilized" plasma, have been demonstrated to be chemically, antigenically, immunologically, and electrophoretically stable immediately after drying and during seven years' storage[2]. Since proteins, enzymes, and vitamins have been preserved by freeze-drying by the pharmaceutical industry, it was postulated that bone tissue could be processed similarly and stored for subsequent grafting.

The procedure consists in freezing the bone and drying it under a high vacuum. In the majority of biologicals the solute remains evenly distributed without becoming concentrated as the frozen solvent sublimates. As a result the dried bone is porous, with a friable, spongelike structure. Cancellous bone crumbles easily, while cortical bone is rigid and inelastic. Both types of bone are reconstituted to normal physical properties in fluid, whether it be saline, plasma, or the patient's hematoma. The process is continuous and can be conducted in the final container in which the material is sealed under vacuum. The average deep-freeze temperature for bone preservation is about −20 degrees to −30 degrees centigrade, and many units have operating temperatures that are even warmer. According to Gersh, about −20 degrees centigrade is well below the freezing point of water containing the normal proportion of body salts[3]. However, even at this and colder temperatures, bone in doubly sealed bottles undergoes some loss of moisture by evaporation. It is conceivable, therefore, that, as the frozen bone tends to dehydrate, the salt concentration in the tissue may be increased sufficiently to lower still further the freezing point of the bone protein. This dessication will tend to denature protein, not only because of the pH change, but also possibly because of the thawing effect achieved by depressing the tissue freezing point below the average deep-freeze temperature. Denaturation (the internal rearrangement of peptide chains which thereby changes the basic physical prop-

* Read at the Annual Meeting of The American Academy of Orthopaedic Surgeons, Chicago, Illinois, January 30, 1951.

FIG. 1

Photomicrograph (× 14, hematoxylin and eosin stain) of eight-day freeze-dried homogenous graft, demonstrating profuse intramedullary trabecular new-bone formation which is in intimate contact with the graft. *A*: Graft, *B* and *C*: Host.

FIG. 2

Photomicrograph (× 275, hematoxylin and eosin stain) of eight-day freeze-dried homogenous graft, demonstrating intramedullary reactive bone formation against the graft surface.

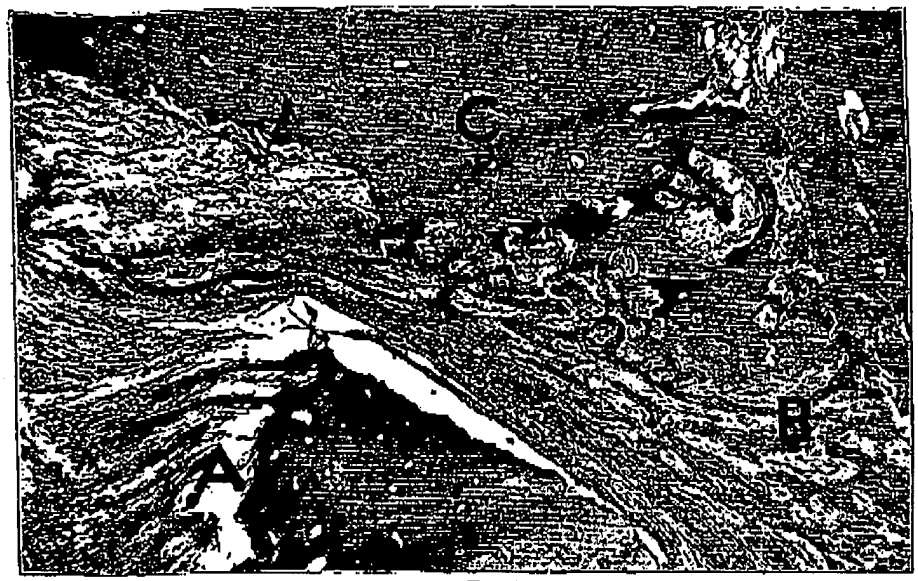

FIG. 3

Photomicrograph (× 40, hematoxylin and eosin stain) of fourteen-day freeze-dried homogenous graft, demonstrating both intramedullary and periosteal reactive bone formation. *A:* Periosteal new bone, *B:* Intramedullary reactive bone, *C:* Graft.

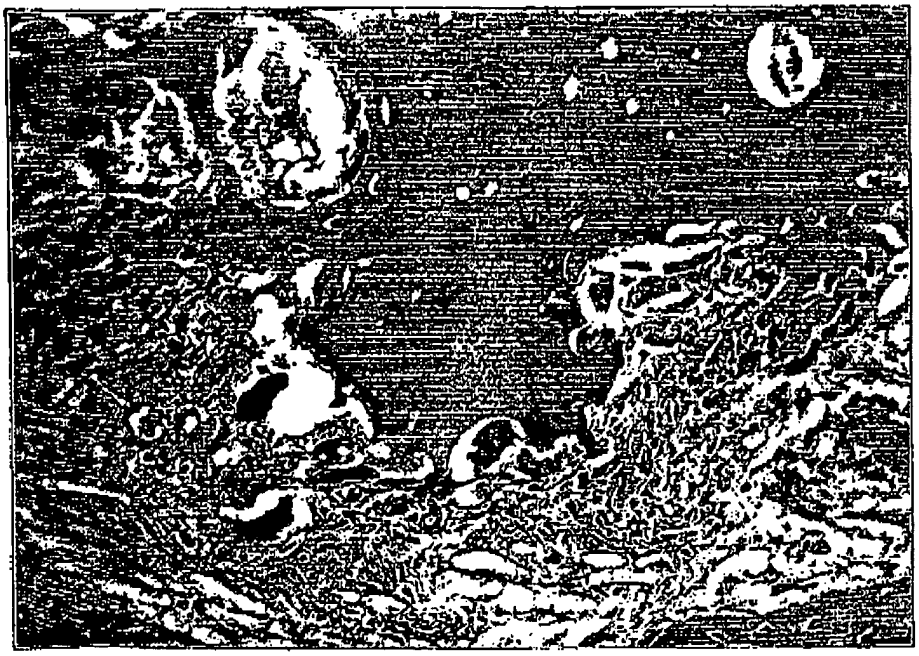

FIG. 4

High-power view (× 275, hematoxylin and eosin stain) of intramedullary reactive bone against graft (magnification of *B* in Fig. 3).

erties of the protein)[8] of osseous protein is considered a factor retarding revascularization of a graft; an example of this is the slow revascularization of a piece of boiled bone.

These factors seem to be corroborated by clinical observations. Wilson observed

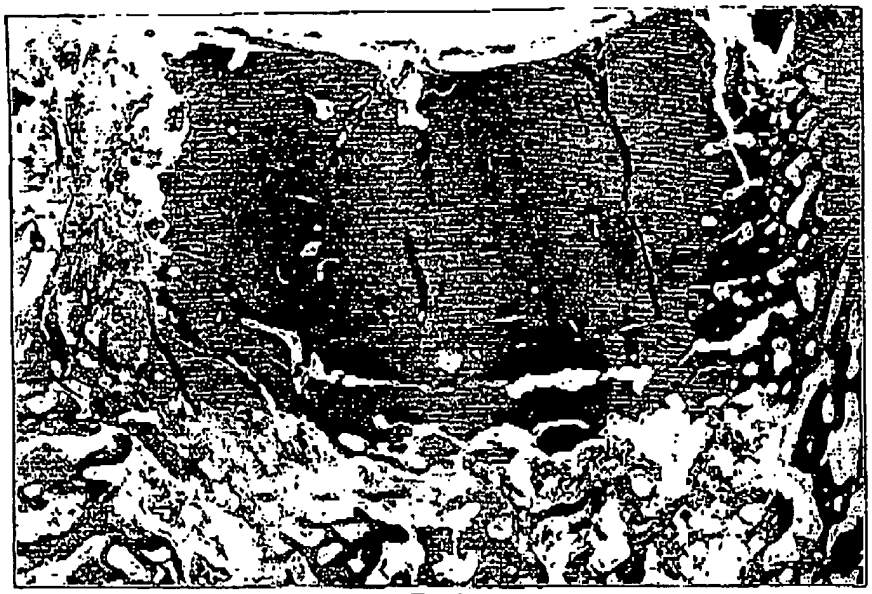

FIG. 5

Photomicrograph (× 14, hematoxylin and eosin stain) of thirty-five-day freeze-dried homogenous graft, demonstrating profuse intramedullary maturing reactive bone.

FIG. 6

Photomicrograph (× 14, hematoxylin and eosin stain) of freeze-dried homogenous graft after 150 days. The remodeling is completed; moderate porosity is the only evidence of previous graft surgery.

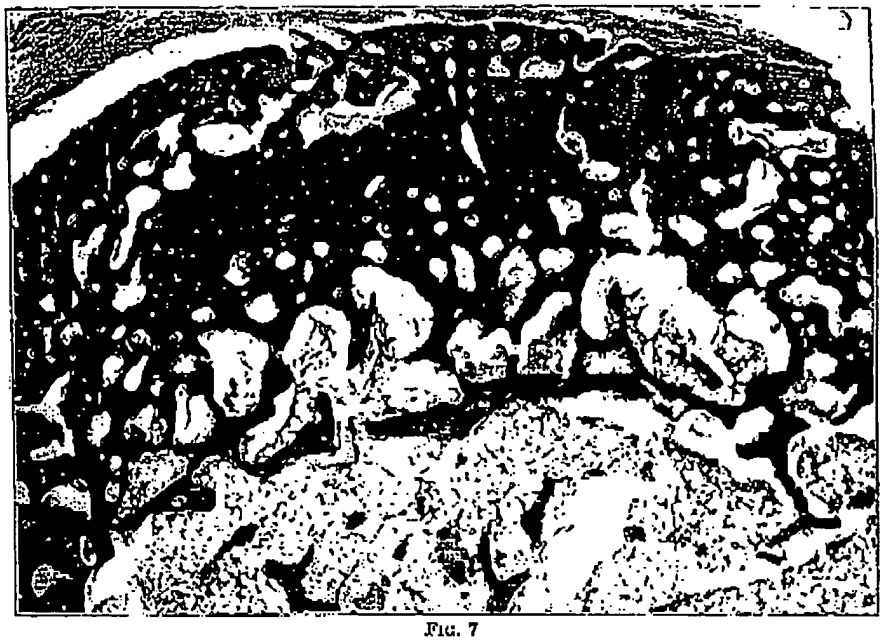

FIG. 7

Photomicrograph (× 14, hematoxylin and eosin stain) of seventy-day freeze-dried homogenous graft, demonstrating confluent vascular channels which have formed a sinusoid in the area of contact between graft and bone.

that frozen bone stored for one year in a single jar does not seem to give as satisfactory a clinical result as bone preserved for shorter periods of time. Speed and Smith noted gross changes,—namely, discoloration, dryness, and loss of elasticity in bone frozen in doubly sealed jars stored for periods as short as three months.

It was decided that study would be devoted to the acceptance and incorporation of freeze-dried bone in animal experiments. The controls consisted of fresh autogenous and frozen homogenous grafts and, in the remaining control animals, of a bone defect the size of the grafts, which was not treated by grafting.

METHODS OF GRAFT PRESERVATION

Freeze-Drying

The technique of drying from the frozen state has been perfected by Flosdorf *.

Freeze-dried bone was prepared by slow freezing at −15 degrees centigrade for four days. Quick-freezing to low temperatures (below −55 degrees centigrade) would have been ideal; however, we did not realize this until the experiment was well advanced. The specimens were placed in dry ice for the eight-hour period of shipment to the proper laboratories. The bone was encased in a block of ice and dried in a Stokes machine under high vacuum at −40 degrees centigrade. The procedure required approximately fourteen days for completion. The shortest approximate storage time was three days, the longest 259, and the average 122 days. This bone was stored on the shelf at room temperature.

Freezing

The frozen bone was stored in single sterile, sealed jars at −15 degrees centigrade. The shortest approximate storage time was five days, the longest fifty, and the average twenty-nine days.

* E. W. Flosdorf, Director, Research and Development Division, F. J. Stokes Machine Company.

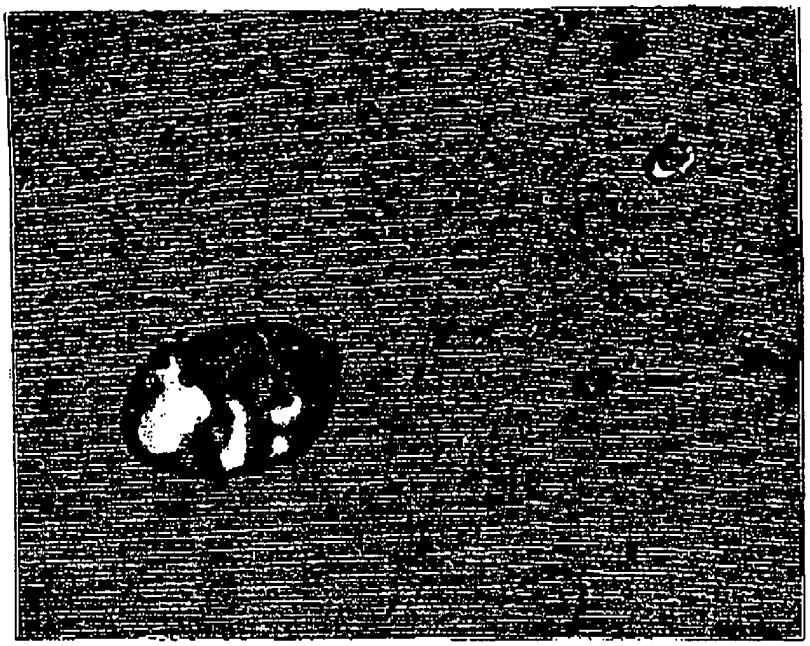

FIG. 9

Photomicrograph (×250, hematoxylin and eosin stain) of freeze-dried homogenous graft after 225 days, demonstrating the concentrically deposited new bone filling the previously dilated vascular channels of the graft.

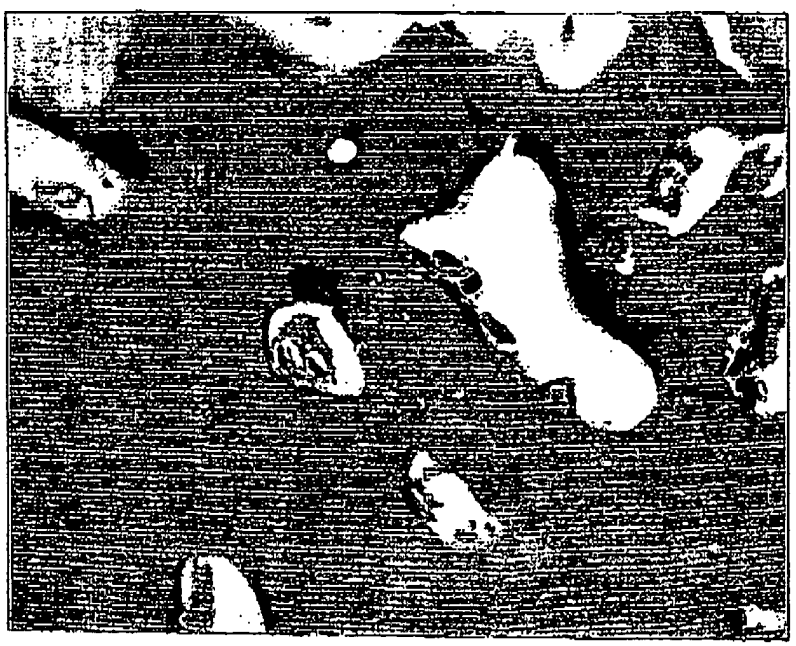

FIG. 8

Photomicrograph (× 250, hematoxylin and eosin stain) showing contact site of seventy-day freeze-dried homogenous graft and demonstrating enlarging vascular channels. Note the large cells lining the channel periphery.

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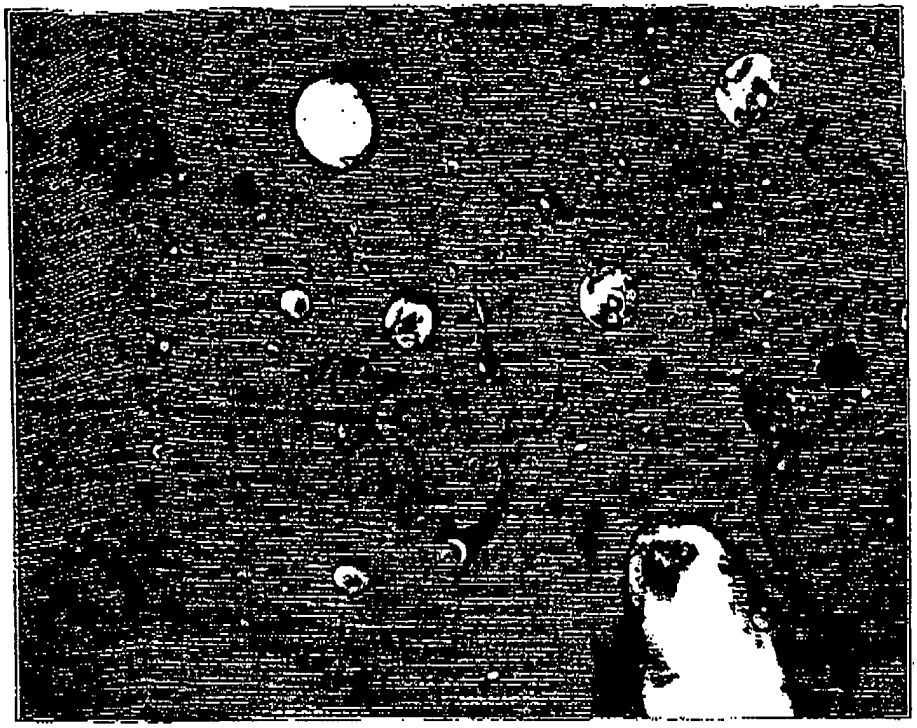

FIG. 10

Photomicrograph (× 275, hematoxylin and eosin stain) of fresh autogenous graft after 287 days, demonstrating new bone filling the previously dilated vascular channels. A considerable amount of dead bone still remains, which will be slowly eliminated by appositional bone substitution, — that is, creeping substitution.

EXPERIMENTAL PROCEDURE

Adult mongrel dogs with an average weight of forty-one pounds were maintained on a standard diet of frozen horse meat and Purina dog chow for the duration of the experiment.

The operative procedure consisted of inlaying a cortical graft into the extensor surface of the mid-shaft of the radius in which a standard-size defect had been made *. The grafts measured 6 to 8 centimeters by 0.5 to 1.0 centimeter. This method secured immobilization without special internal or external fixation. As a control, a fresh autogenous graft was removed and replaced, reversing the direction of the longitudinal axis. No attempt was made to cover the graft with periosteum. As an added control, the standard-size defect was made without implanting a graft.

In the total of forty-four dogs, freeze-dried homogenous grafts were placed in eleven, frozen homogenous in twelve, fresh autogenous in thirteen, and no grafts were used in eight. The animals were sacrificed at about eight, fourteen, twenty-four, thirty-five, sixty, ninety, 120, and 200 or more days.

RESULTS AND INTERPRETATIONS

The response of the host to all grafts has been divided into the following phases:

1. *Reactive Bone Formation:* This is the response of the host to injury and is dem-

* Early operative work was done by Lieutenant, j.g., A. G. Marrangoni, Medical Corps, United States Naval Reserve (Inactive).

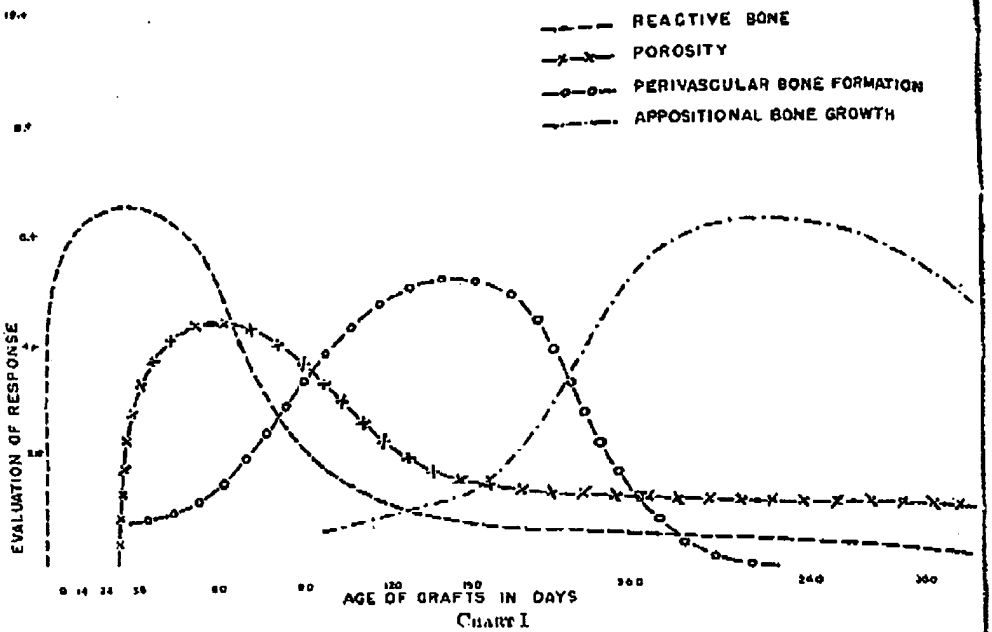

Chart I

A graphic summary of the four phases of graft incorporation by the host. Plus units are used to denote the intensity of response which is numerically evaluated on the graph ordinate.

onstrated by profuse intramedullary and periosteal trabecular bone formation in which can be seen a considerable number of multinuclear giant cells. There was a notable intramedullary reaction in the host as early as at eight days (Figs. 1 and 2); however, it was most marked at fourteen days (Figs. 3 and 4). As early as the twenty-fourth day the reactive bone appeared to be maturing as the trabeculae thickened and the intramedullary spaces were noticeably widened. The intramedullary cavity was still filled with reactive bone (Fig. 5). This type of reactive bone formation seals the graft in a form of fixation to the host. The next phase is remodeling, which may be defined as the restoration of the anatomical continuity of the intramedullary cavity, of the periosteum, and the obliteration of the contact sites by mature bone (Fig. 6). Completed remodeling represents the terminal phase of reactive bone formation. Usually this reactive response was completed between ninety and 120 days.

2. *Revascularization:* This is the intense vascular phenomenon associated with porosity of the graft and accompanied by a very similar response in the host. It was consistently evident as early as the thirty-fifth day. Through mediation of mononuclear and multinuclear cellular de-ossification the original Haversian vascular channels are apparently enlarged to form a series of confluent sinusoids (Figs. 7 and 8). This vascular response follows the pre-existing channels throughout the graft as if it were a trellis. Revascularization of the graft and host and the resultant porosity was most marked at between sixty and ninety days (Fig. 7). It was still markedly evident at 120 days. After this period the vascular channels of both the host and the graft persisted in slightly increased number and size throughout the period of 260 days.

3. *Perivascular Bone Formation:* New mature-appearing bone was laid down concentrically around the vascular sinusoids. The lumen of these channels gradually decreased in size in direct proportion to the amount of new mature bone which was formed (Fig. 9). It was noted that the concentric lamellae which replaced the porosity resulted in the formation of uniform bone.

4. *Appositional Bone Substitution:* The new bone which was laid down in a form provided by the pattern of vascularization in some instances did not include all of the graft substance. Therefore, some irregular elements of the dead graft remained. It may be that these will be eliminated by appositional growth in the manner by which normal bone ordinarily sustains itself. This is not a dramatic tissue response but a slowly progressive one; this process has been designated as creeping substitution (Fig. 10).

The histological phases previously described are not clean-cut or sharply differentiated but blend one into another as demonstrated in Figure 11. The manner of graft incorporation did not appear to differ in any of the transplants. The notable differences were those of rate. Frozen homogenous grafts, particularly in the early stages, appeared to be incorporated more slowly than the freeze-dried bone. Apparently freeze-dried bone is incorporated by the host at a slightly slower rate although in a manner identical to fresh autogenous bone. Defects implanted with frozen homogenous grafts healed in an identical manner although probably at a slower rate than those grafted with freeze-dried homogenous bone. Except for these differences in rate, the healing of all defects in which grafts were used was roughly comparable to that of the bone defects in which no grafting was done. However, it was noted that in the untreated bone defect there was a preponderance of cartilage over bone after 142 days.

Of interest in this small series is the suggestion that in certain respects, that is, the rate of revascularization and early new-bone formation about the graft, freeze-dried bone is more rapidly transformed into new bone than is frozen bone. While the difference in rate is probably not clinically significant, it is significant enough to warrant further experimental explorations. The possibility that bone protein is changed by freezing or freeze-drying may partly explain the slight delay in incorporation, as compared to fresh autogenous bone; this is currently being investigated.

The results of these operative procedures seemed to justify clinical use. Accordingly six patients were treated with freeze-dried homogenous grafts at the United States Naval Hospital, Bethesda, Maryland. The end results will be reported at a later date. Early results in patients both roentgenographically and clinically, have supported experimental findings in the animal.

These results should form the basis for extensive investigations on the practicality of a large bone bank which would meet the needs of the majority of naval hospitals. The procurement and use of such grafts should be under the control of a central authority.

CONCLUSIONS

1. Freeze-dried homogenous bone grafts measuring 6 to 8 centimeters by 0.5 to 1.0 centimeter were grafted into the radii of dogs. It was found that they were incorporated in the same manner as fresh autogenous bone grafts but at a slightly slower rate. The freeze-dried bone was somewhat superior to frozen bone in regard to the rate of healing in the early phases.

2. Except for the previously mentioned variations in the rate of healing, all the grafts were incorporated in a manner roughly comparable to the healing of the bone defect of the same size in which grafts were not implanted.

3. The advantages of storage at room temperatures, the potentially increased storage periods, the ease of transportation, and initial clinical success suggest that freeze-dried homogenous bone is suitable for use in bone banks.

NOTE: Photomicrographs are produced by courtesy of *Armed Forces Institute of Pathology* and *Photographic Department, Naval Medical School, Bethesda, Maryland.*

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DISCUSSION

DR. PAUL R. LIPSCOMB, ROCHESTER, MINNESOTA: Codman made the statement, "Give me something different for there is a chance of its being better". The authors of this paper have given us a different method of preserving bone and, although it is admittedly better for the long-term preservation of the proteins, enzymes, and salts of stored bone, the question arises, is this method better from a practical and economical standpoint? The authors could detect no appreciable difference in the results when bone preserved by freeze-drying was used from those when bone frozen for an average of twenty-nine days was used.

The authors stated that denaturation of proteins is detrimental to acceptance of any graft by the host, for it evokes a foreign-body response. This statement to me seems logical, and yet Reynolds and Oliver stated that bone preserved in merthiolate is just as satisfactory as bone preserved by freezing. Merthiolate and most other antiseptics derive their power from the fact that they are protein coagulants. At the Mayo Clinic we have noted no ill effects which could be attributed to protein denaturation when we used bone preserved by freezing alone, although we have been hesitant to use bone which has been preserved for more than three to six months. It is obvious that, if protein denaturation does not affect the fate of the transplant, those of us who are going to the trouble and expense of minimizing protein denaturation by using frozen bone are doing so needlessly. Likewise, if this is the case, and I doubt it, the complicated and expensive process of freeze-drying whereby the denaturation of protein is absolutely stopped would not be justified. Although the freeze-drying or lyophilization process of the preservation of bone is from a theoretical standpoint the best method yet developed for preserving bone, one wonders whether, from a practical and economical standpoint, the method is sufficiently better to justify its use in civilian practice in preference to the other two methods which have been advocated. In military practice, when the procurement of the bone is under a central authority and the bank would necessarily be large, this method of preservation of bone would be ideal provided that such bone is definitely superior to that preserved by antiseptics. Likewise, should the Red Cross or the pharmaceutical companies ever dispense homogenous bone by methods of distribution similar to those now being used for plasma, this process would probably be feasible.

Those who believe that some osteoblasts actually live when transplanted in fresh autogenous grafts argue that autogenous transplants are superior to homogenous transplants for this reason. This argument may be settled by a comparison of lyophilized autogenous transplants with fresh autogenous transplants. In the first instance, the salts, proteins, and enzymes and presumably the tissue specificity would be unchanged, but all living cells would be destroyed, whereas in the fresh autogenous graft, living cells would be retained. It may be that the freeze-drying process, since it destroys the cells of bone but, as far as we know, does not alter the chemistry *in vitro*, will give us another valuable tool for the study of osteogenesis. I would like to ask the authors if they have made any observations suggestive of this.

The authors are to be congratulated for presenting to us another method of preserving bone and for their scientific and long-term analysis of the efficacy of bone so preserved.

DR. JAMES B. WEAVER, KANSAS CITY, KANSAS: I feel that Captain Kreuz and his associates have done a well thought-out, well-planned, and well-executed piece of research work.

There are two things which I would like to have clarified if Lieutenant Hyatt will do so. No mention has been made as to the effect on the sterility of these grafts by freeze-drying. Does freeze-drying kill bacteria or not? I assume that it does not.

The statement was made that the end product of this process is porous and crumbly. We have all had the experience of relying upon our graft for a certain amount of internal fixation, yet frequently at the site of stress and strain we get absorption of the graft and non-union. If freeze-dried bone is more friable, then what are we to expect if we use this bone for internal fixation?

LIEUTENANT HYATT (closing): In reference to Dr. Lipscomb's comments, we feel that the theoretical protein difference is notoriously difficult to evaluate in experimental animals, and will probably best be determined by objective observations over a long period of time. We do not recommend freeze-drying as a method for general use. It will be a closely controlled clinical research project, and in five to seven years, we may have an answer.

With reference to protein products in relation to their denaturation by methods of preservation, we are now doing some work which is an evaluation utilizing enzyme digestion of the denatured protein products. In this manner the degree of denaturation of bone protein resulting from a method of preservation may be numerically evaluated.

(*Continued on page 888*)